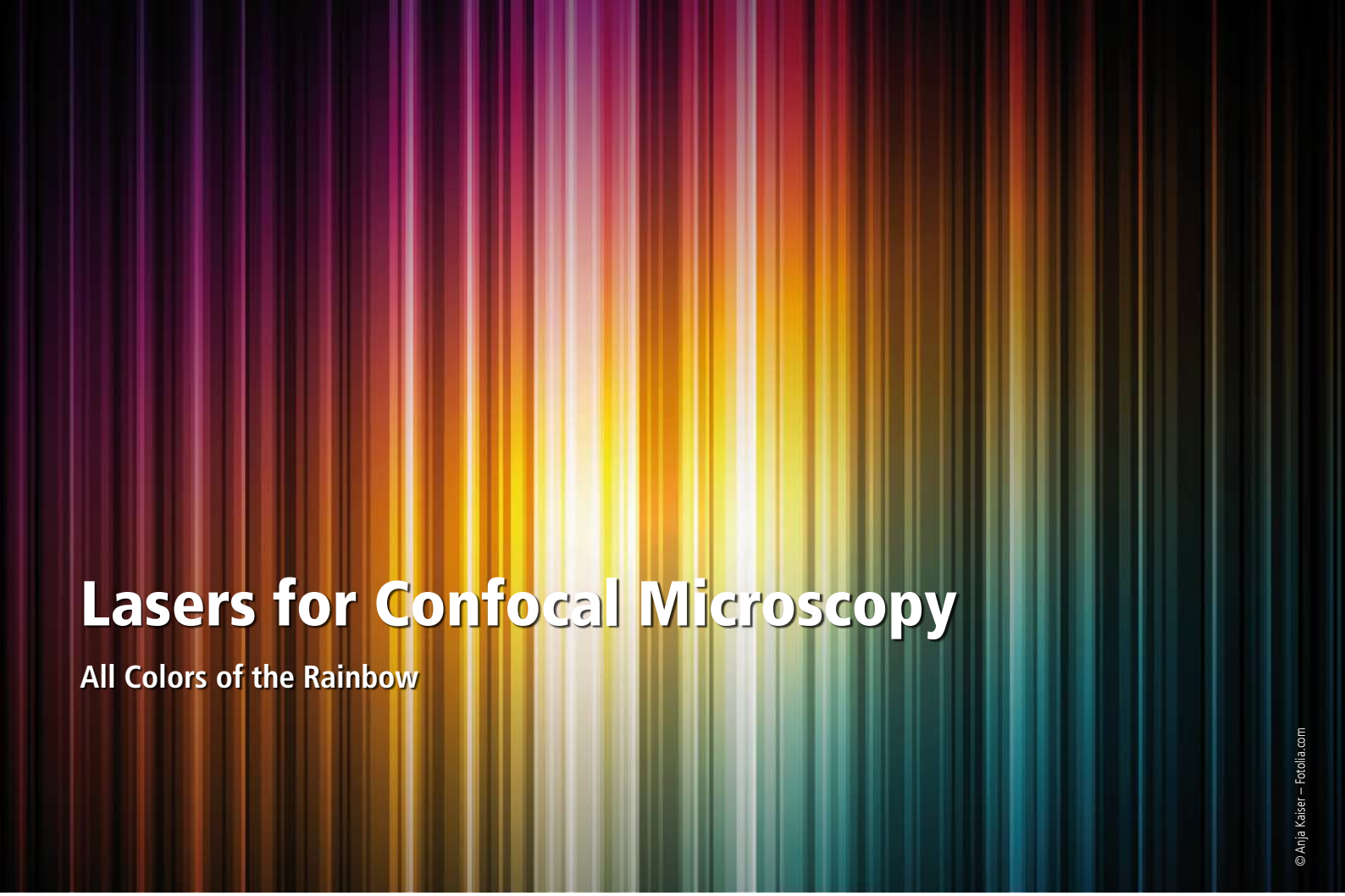

Lasers for Confocal Microscopy

All Colors of the Rainbow

Easy-to-use new lasers are more and more replacing bulky legacy laser systems. This new generation of lasers provides flexibility, easy operation and is all in all more convenient to use. These novel approaches offer also new possibilities – supporting the rapid development of new microscopy techniques. Here we will review different laser types and their fields of application.

Keywords

Laser, Microscopy, Fluorescence, Multicolor

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In conventional wide-field microscopes arc lamps (e.g. Xe, Hg) are used for illumination. They cover the complete visible range and thus can excite a broad range of dyes. Although they have powers of 100 Watt and more, their radiance (flux density per unit solid viewing angle) is not high enough to give satisfying results when applied to point scanning or spinning disk confocal microscopy. Interestingly, when Marvin Minsky invented the confocal microscope in 1957, he did not use a laser for illumination (it was still to be invented) but a zirconium arc lamp. The first commercially available laser scanning confocal microscope used an air-cooled argon laser for excitation. In comparison with other light sources lasers provide a high brightness, a small divergence and they are highly monochromatic, properties which make them ideal light sources for confocal microscopy.

Gas Lasers such as Ar-ion, Kr-ion and others are still widely used for microscopy as for a long time only these were able to provide the colors and beam quality required. Occasionally dye lasers are employed which consist of a solution of fluorescent dye pumped by a suitable laser source. These are able to cover a broad spectral range but are rather cumbersome to handle.

Since a few years diode and DPSS lasers are available, that fulfill the requirements for microscopy such as spectral stability and beam quality. These compact and easy to use lasers are currently replacing the antiquated laser systems. DPSS lasers are diode-pumped solid state lasers with red or near infrared laser diodes integrated in a resonator. SHG converts the (infra) red light into the visible range. Diode lasers use conventional laser diodes to directly produce the output wavelength; however for years, it was impossible to directly generate wavelengths in the blue and green spectral range. In 1998 the emergence of the semiconductor material Gallium Nitride (originally developed for the optical data storage industry) facilitated the direct generation of blue wavelengths. These new diode lasers, above all 488 nm, were quickly established. Figure 1 shows a confocal image of rat brain axons acquired with the iBeam smart 488 nm diode laser. This year another big step was taken: 515 nm is now available as direct diode technology, finally filling the "green gap".

Intensity Modulation

Intensity modulation or fast wavelength switching with gas or DPSS lasers requires additional acousto- or electrooptical modulators. In contrast, diode lasers can be directly modulated at high frequencies (e.g. the iBeam smart with up to 250 MHz). Fast switching is generally required to blank the laser during fly-back in scanning systems to re-

duce photobleaching. Furthermore, this feature is advantageous for microscopy techniques such as FRAP (Fluorescence Recovery After Photobleaching) or CLEM (Controlled Light Exposure Microscopy), techniques that require rapid switching of wavelengths and intensity modulation.

Multicolor Applications

For many applications more than one excitation wavelength is required in order to localize different structures with respect to each other. Usually, not more than two to four stains are used in one sample. An example to extend four colors in order to discriminate up to 90 colors is the so-called "Brainbow"-technique where individual neurons express stochastic combinations of three fluorescent proteins. For multicolor experiments, two different approaches are available: first, the use of white-light lasers and tunable lasers, respectively. In the second approach several lasers are combined in one multi-laser system, whose output is then coupled into the microscope.

Multi-laser Engines

As for the latter, stable alignment of multiple laser sources is still an issue which is crucial for a convenient use of such systems. Quite often "breadboard approaches" are employed, where lasers are mounted on an optical table and combined via dichroic mirrors. These approaches, however, are prone to misalignment due to environmental influences. An alternative to that is the iChrome MLE which combines up to four individual lasers (wavelengths from 405 nm to 640 nm can be selected) within one box (fig. 2). The individual lasers can be switched on and off independently via TTL or using an integrated acousto-optical modulator (AOM) for versions including a DPSS laser. The system overcomes the aforementioned stability issues by an auto-calibration technology, which guarantees high and constant optical output levels. Depending on the lasers included, those systems emit single-line power levels of up to 50 mW after fiber delivery and can be easily integrated into existing setups. Figure 3 shows a maximum projection of a mouse kidney section acquired with the iChrome MLE.

Tunable Light Sources

The second possibility for multi-color applications is to use tunable light sources, which cover the complete visible range such as Optical Parametric Oscillators (OPO), super continuum light sources (also referred to as white light sources) or tunable visible laser sources. OPOs have a broad tuning range (~300 nm) and high maximum output powers (~ 1 W) but are rather complex systems, frequently pumped by Titanium:Sapphire lasers. White light sources on the other hand generate a supercontinuum, typically from 460 nm to 2 µm, by using a photonic crystal fiber (PCF) and emit all colors simultaneously. Narrow band filters or an AOTF are required for wavelength selection. The several Watt output powers are distributed over the complete wavelength range, leading to powers in the range of several mW/nm. The third concept is a tunable light source which allows setting all wavelengths in the visible range. In this approach narrow bandwidth pulses are not filtered from a supercontinuum; instead ultrashort laser pulses generate the new colors by four wave mixing in a highly nonlinear fiber. SHG converts the output of the fiber, generating laser pulses tunable in the visible range (from 488 to 640 nm). This approach is realized in the iChrome TVIS. The pulsed operation of the system (3.5 ps) makes it ideally suited for fluorescence lifetime measurements (FLIM). A FLIM image of dsRed stained mitochondria is shown in figure 4.

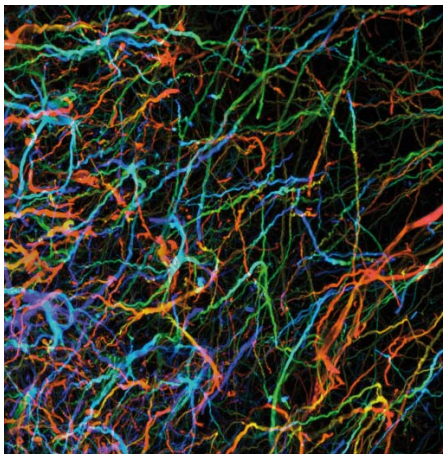

Fig. 1: Left: Color-coded z-projection of axons in rat brain, acquired with the iMIC Andromeda (Till Photonics) and the iBeam smart 488 nm. Right: iBeam smart at several wavelengths.

Fig. 2: Multi-laser engine iChrome MLE

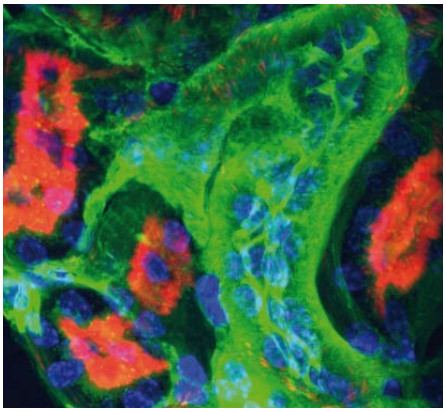

Fig. 3: Maximum projection of a z-stack of a mouse kidney section excited at 405 nm (DAPI), 488 nm (Alexa 488) and 561 nm (Alexa 568).

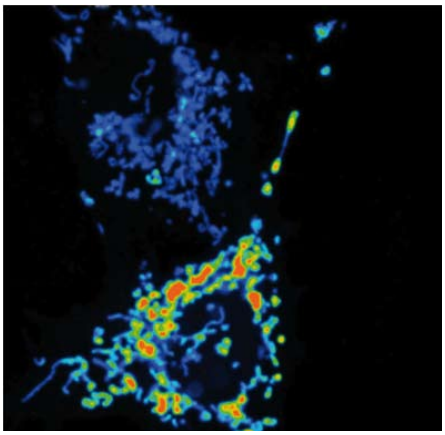

Fig. 4: FLIM measurement of mitochondria (dsRed) in Cos7 cells acquired with the iChrome TVIS at 550 nm excitation (Courtesy of Dr. Roland Nitschke, Life Imaging Center, Freiburg).

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